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13. ABSTRACT

Thrombospondin-1 is an extracellular matrix protein that has been shown to modulate the response of endothelial cells to growth factors. Specifically the protein suppresses endothelial cell proliferation, migration and angiogenesis in vivo. This grant allowed us to ascertain the significance of thrombospondin for the suppression of the vasculature in mammary tumors. Utilizing null and TSP-1 overexpressing mice, we determined that the protein plays a major role in the size, architecture, and number of vessels in mammary tumors. We have also found that both CD-36 and $\alpha_3\beta_1$ are critical signaling receptors for the anti-angiogenic effects mediated by TSP-1.

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FOREWORD

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INTRODUCTION

In this final report, we are pleased to communicate that all tasks have been concluded as proposed. A total of 6 manuscripts have been published (see page 9 of this report), 2 are currently under review, and 2 are in preparation. In addition, two book chapters have been written based on this work. Finally, our results have been presented in 4 National meetings and 2 International meetings all as invited speaker (year 1999 alone). Overall, we are pleased with the outcome and determined to continue this line of investigation to ascertain the intracellular pathways that mediate thrombospondin-1 suppression of vascular growth.

BODY

EXPERIMENTAL METHODS / AIMS (as presented in original proposal)

A. Is the lack of TSP1 associated with growth and metastasis of malignant tumors?

Examine the progression of the vascular bed in mammary tumors of TSP1-deficient mice.

Experimental Design/Methodology:

1. Generate mammary tumors in TSP1-deficient (tsp/tsp-) mice by mating of TSP1 knock-out homozygotes with mice carrying the MMTV c-*neu* transgene

2. Analysis of the vascular bed, as well as rate of capillary extension/mm2 of neoplastic tissue will be obtained by: a) confocal laser analysis coupled with three-dimensional reconstruction, b) determination of hemoglobin and c) endothelial cell markers. Values obtained from TSP1-deficient and from control *neu* animals will be compared.

Overall growth of the tumors and rate of metastasis will also be directly assessed and correlated with control values. Data from these experiments will concurrently provide important information on the relationship between capillary density and tumor expansion.

Determine whether exogenous TSP1 can revert/rescue the vascular phenotype of induced tumors in TSP1-deficient mice.

Experimental Design/Methodology:

1. Slow-release pellets of TSP1 protein will be implanted in the mammary fat pads of TSP1-deficient mice carrying the MMTVc-*neu* transgene.

2. Vascular progression in tumors will be determined and compared to control-*neu* mice.

3. In addition, the localization of exogenous TSP1 protein and its half life in tumors will be assessed to gain information on the fate of exogenous TSP1 in mammary tumors.

B. What are the specific effects of TSP1 on endothelial cells engaged in angiogenesis?

Investigate the specific effect(s) of TSP1 on endothelial cells engaged in the angiogenic response.

1. Endothelial cells (EC) from normal mouse mammary gland and from mammary tumors will be isolated and characterized for their proliferation rate, secretory profile, and angiogenic potential.

2. Exogenous TSP1 will be added to EC at confluence or to cells undergoing angiogenesis *in vitro*. These experiments will be performed in both tumor-derived as well as control cells. We will determine the effect of this addition on: a) proliferation; b) migration; c) chemotaxis; and d) expression of extracellular matrix-associated molecules.

Identify the cell surface receptor(s) involved in mediating cellular responses to TSP1.

1. The presence of TSP receptors will be assessed in cultures of confluent EC, as well as in angiogenic cultures, by direct binding assays.

2. Modulation of receptor number will be analyzed after addition of TSP1.

3. Neutralizing experiments with specific anti-TSP receptor antibodies will be performed to determine which of the five recognized receptors mediates an anti-angiogenic response.

RESULTS

STATEMENT OF WORK (as presented in original proposal)

Task 1, Examine the progression of the vascular bed in mammary tumors of TSP1-deficient mice, Months 1-24.

a. Generate TSP1-deficient mice containing mammary tumors

b. Tumors from experimental and from control animals will be harvested and measured

c. Analyze the frame-work of capillaries in both experimental settings by morphometry

d. Analyze the frame-work of capillaries in both experimental settings using a biochemical strategy.

Task 2, Determine whether exogenous TSP1 can revert/rescue the vascular phenotype of induced tumors in TSP1-deficient mice, Months 12-30.

a. Preliminary experiments:
Determine the rate of release of [125I]-TSP1 and determine its half life in mammary tumors. Adjust experimental conditions to accommodate these results.

b. Implant capsules containing TSP1 into the mammary tumors of TSP1-deficient mice

c. Determine the effect of TSP1 on the vascular density of mammary tumors as performed in Task #1 (c and d).

Task 3, Investigate the specific effect(s) of TSP1 on endothelial cells engaged in the angiogenic response, Months 23-40.

a. Isolate and characterize endothelial cells from normal and from tumor containing mammary glands for their proliferation rate, secretory profile, and angiogenic potential
b. Add TSP1 to endothelial cells from normal and tumor of the mammary gland and assess a) proliferation; b) migration; c) chemotaxis; and d) expression of extracellular matrix-associated molecules.

Task 4, Identify the cell surface receptor(s) involved in mediating cellular responses to TSP1, Months 30-48.

a. Characterize and compare the spectrum of TSP1 receptors expressed by normal and tumor-derived endothelial cells at confluence and after they organize into cords and tubes.
b. Perform Western blots to determine whether one or more of the previously characterized receptors is modulated
The presence of TSP receptors will be assessed in cultures of confluent EC, as well as in angiogenic cultures, by direct binding assays.
Modulation of receptor number will be analyzed after addition of TSP1.
Neutralizing experiments with specific antibodies will be performed to determine which of the five receptors identified for TSP1 mediates an anti-angiogenic response.

SUMMARY OF ACCOMPLISHMENTS -

Nov. 1994 - Nov. 1998

Task 1-	Months 1-24	Completed
Task 2-	Months 12-30	Completed
Task 3-	Months 23-40	In progress
Task 4.	Months 30-48	In progress

Dec. 1998 - Nov 1999 - Final Report

Task 3 - We have now concluded the experiments that aimed to examine the effects of TSP-1 on the production of extracellular matrix proteins by endothelial cells. As will be discussed (point-to-point discussion) we found no alterations in the pattern of secreted proteins when exogenous TSP1 were added to endothelial cultures. To expand on this aim (although not proposed in the original grant), we decided to evaluate the effect of TSP-1 on the matrix metalloproteinases 2 and 9. Our results revealed that TSP-1 increases pro-MMP-2 levels when added to endothelial cultures. It is unclear if this effect is due to an increase in mRNA or if TSP-1 blocks further processing of this MMP. If so, TSP1 would limit the availability of active MMP-2 in the immediate extracellular environment and explain the suppression of migration seen in the presence of TSP1. We are actively pursuing this line of investigation.

Task 4 - This task has now been concluded. During this year we found, together with the laboratory of Dr. David Roberts, that TSP1 binds the integrin alpha3 beta 1. Binding to

this integrin modulates the responses of endothelial cells to TSP1. Nonetheless, our data (as presented in previous reports) agrees with that of other laboratories that the anti-angiogenic response of TSP-1 is mediated by CD-36. The results related to CD-36 were presented in the last two reports.

TASKS CONCLUDED THIS YEAR - POINT-TO POINT DISCUSSION

Task 3, Investigate the specific effect(s) of TSP1 on endothelial cells engaged in the angiogenic response, Months 23-40.

During year 4 we concluded and expanded this task.

Addition of TSP1 to endothelial cell cultures was performed as follows: endothelial cell cultures were allowed to spread for 24hrs and were later exposed to increasing concentration of TSP-1 for 24, 48, and 72hrs. Controls included: BSA, fibronectin, and heated-TSP-1. Endothelial proteins were labeled with [3H]-proline and secreted proteins from conditioned media were concentrated by dialysis and lyophilization. Examination of proteins was done by SDS-PAGE. Proteinaceous material representing equal counts (100kcpm) was loaded in each lane. Experiments were performed in triplicate. Initial assays were done using bovine aortic endothelial cultures and were later repeated using human microvascular endothelial cells.

As presented in Figure 1, we found that TSP1 did not alter the pattern of secreted proteins by endothelial cells. The rationale behind the experiments was to ascertain whether TSP-1 alters the immediate microenvironment of the migrating endothelium and thus suppresses angiogenesis. The answer to our question based on these data appears to be negative.

To follow up on the effects of TSP-1 on the extracellular matrix, we decided to expand this task and evaluate the effect of TSP1 on gelatinases. Using zymography we found a reproducible and dose-dependent increase in pro-MMP-2 on endothelial cell cultures exposed to TSP-1. Heated TSP-1 had no effect. More recently we found that TSP-1 can suppress the processing of pro-MMP-2 to active MMP-2 thus restricting the levels of catalytically active enzyme. Since endothelial migration during angiogenesis requires digestion of extracellular matrix proteins by MMPs restriction of active proteases can restrain the ability of cells to invade the stroma. Indeed TSP1 has been shown to suppress endothelial migration in several assays. It appears that regulation of MMPs can be an attractive mechanism for the effects of TSP-1 on migration of endothelial cells. (These data are not represented as figures, since these experiments were not part of the original proposal).

Task 4, Identify the cell surface receptor(s) involved in mediating cellular responses to TSP1- Months 30-48.

Other results related to this task have been presented in the last two reports.

Several regions in TSP-1 have been reported to regulate endothelial cell behavior and inhibit angiogenesis by interacting with CD36 or heparan sulfate proteoglycan receptors. We have found through investigations proposed in this task that binding of TSP1 to a3ß1 can modulate angiogenesis. Although we found that confluent endothelial cells do not spread on a TSP1 substrates, efficient spreading on TSP1 substrates of

endothelial cells maintained without cell-cell contact is mediated by the a3ß1 integrin (Figures 2 and 3). We found that in solution, an a3ß1 integrin-binding peptide from TSP1 inhibits proliferation on sparse endothelial cultures, but the same peptide immobilized on the substratum promoted proliferation (Figures 4 and 5). Intact TSP1 also selectively promotes endothelial cell proliferaion when immobilized on the substratum. The TSP-1 peptide, when added in solution, specifically inhibited migration of endothelial cells into scratch wounds and inhibited angiogenesis in the chick chorioallantoic membrane assay (data not shown). Thus, recognition of immobilized TSP1 by a3ß1 integrin may increase endothelial cell proliferation and motility during wound repair and angiogenesis. Together our previous results with CD-36 and the present data suggest that the expression of specific receptors and the presentation of TSP-1 is essential to its anti-angiogenic properties.

CONCLUSIONS

From the activities performed during the first quarter of year 4, these are our conclusions:

1) TSP-1 is capable of modulating the extracellular microenvironment by limiting the catalytic activity of MMP-2;
2) The mechanism by which TSP1 suppresses angiogenesis is likely to include a combination of effects related to inhibition of endothelial cell proliferation and attachment. The receptor involved in these effects is likely to be CD-36. Nonetheless, we found that a3ß1 can modulate the responses of endothelial cells to TSP-1. Therefore, the relative expression of TSP-1 receptors on the cell surface is essential in determining the overall effect of TSP-1 in angiogenesis.

PRESENTATIONS AND PUBLICATIONS OF FINDINGS RELATED TO THIS GRANT PROPOSAL

A- PRESENTATIONS IN MEETINGS:

1) Experimental Biology and NAVBO meeting. Speaker and Symposium and co-chair. Washington DC; April 1999.

2) Symposium on Reproductive Angiogenesis, Melbourne, Australia; May, 1999.

3) Extracellular Matrix in Diabetes and Athreosclerosis meeting, Univ. Washington, Seattle; May 1999

4) WFTN, Algarve, Portugal; June, 1999.

5) Gordon Conference on Matrix Metalloproteinases, New Hampshire; August 1999.

6) Gordon Conference on Angiogenesis and Microcirculation, Newport, Rhode Island; August 1999.

B- PUBLICATIONS RELATED TO THIS PROJECT

1. **Iruela-Arispe, M.L.,** Porter, P., Bornstein, P., and Sage, E.H. 1996. Thrombospondin-1, an inhibitor of angiogenesis, is regulated by progesterone in human stromal cells. J. Clin. Invest, 97:403-412

2. Dhanabal, M., Ramchandran, R., Volk, R., Stillman, I.E., Lombardo, M., **Iruela-Arispe, M.L.,** Simons, M., and Sukhatme, V.P. 1999. Endostatin: yeast production, mutants, and anti-tumor effect in renal cell carcinoma. Cancer Res. 59:189-197.

3. **Iruela-Arispe, M.L.,** Lombardo, M., Krutzsch, H.C., Lawler, J., and Roberts, D.D. 1999. Inhibition of angiogenesis by thrombospondin-1 is mediated by two independent regions within the type 1 repeats. Circulation 100:1423-1431.

4. Brown, L.F., Guidi, A.J., Schnitt, S.J., Van De Water, L., **Iruela-Arispe, M.L.**, Yeo, T.K., Tognassi, K., Dvorak, H.F. 1999. Vascular stroma formation in carcinoma in situ, invasive carcinoma, and metastatic carcinoma of the breast. Clin.Cancer Res.5:1041-1056.

5. Zhai, Y., Yu, J., **Iruela-Arispe, L.,** Huang, W.Q., Wang, Z., Hayes, A.J., Lu, J., Jiang, G., Rojas, L., Lippman, M.E., Ni, J., Yu, G.L., and Li, L.Y. Inhibition of angiogenesis and breast cancer xenograft tumor by VEGI, a novel cytokine of the TGF superfamily. Int. J. Cancer 82:131-136.

6. Vazquez, F., Hastings, G., Ortega, M.A., Lane, T.F., Oikemus, S., Lombardo, M. and **Iruela-Arispe, M.L.** 1999. METH1, a human ortholog of ADAMTS, and METH2 are members of a new family of proteins with angio-inhibitory activity. J. Biol. Chem. 274:23349-23357.

7. Ortega, M.A. and **Iruela-Arispe, M.L.** 2000. Thrombospondin-1 is an endogenous regulator of angiogenesis in the mammary gland. J. Cell Biol. Submitted.

8. Chandrasekaran, L., He, Z.C., Sipes, J.M., Krutzch, H.C., **Iruela-Arispe, M.L.**, and Roberts, D.D. 2000. Modulation of endothelial cell behavior and angiogenesis by an a3ß1 integrin-binding peptide from thrombospondin-1. J. Cell Biol. Submitted.

MANUSCRIPTS IN PREPARATION

1. **Iruela-Arispe, M.L.**, Oikemus, S., Hynes, R., and Lawler, J. Thrombospondin null animals exhibit abnormal involution of blood vessels in the mammary gland.

2. **Iruela-Arispe, M.L.,** Lane, T.F., Ortega, M.A., Hynes, R., and Lawler, J. Thrombospondin regulates tumor-induced angiogenesis.

BOOK CHAPTERS

1. **Iruela-Arispe, M.L.**, Vazquez, F., and Ortega, M.A. 1999. Anti-angiogenic domains shared by thrombospondins and ADAM/TS proteins. In: Anti-Cancer Proteins & Drugs: Structure, Function & Design. The New York Academy of Sciences.

2. **Iruela-Arispe, M.L.,** Ortega, M.A., and Vazquez, F. 1999. Anti-angiogenic domain of thrombospondin. In: Angiogenesis in Health and Disease: Basic Mechanisms and Clinical Applications. Gabor Rubany, Editor.

LIST OF PERSONNEL INVOLVED IN THIS PROJECT
For year 4 - 10/98 to 10/99

Dr. Luisa Iruela-Arispe	- Principal Investigator - 20%
Dr. Juan Carlos Rodriguez Manzaneque	- Post-doctoral fellow - 100%
Ms. Leslie Caromile	- Research Assistant - 50%

FIGURE LEGENDS

Figure 1. Effect of TSP1 on extracellular matrix proteins.
Cultures of endothelial cells were plated and allowed to attach for 16h in the presence of fetal calf serum. Media was then removed and cells were cultured in the presence or absence of increasing amounts of TSP1 and labeled with [3H]-proline in the absence of serum. Cultures were incubated for 24hs and conditioned media was removed, dialyzed and subjected to SDS-PAGE.

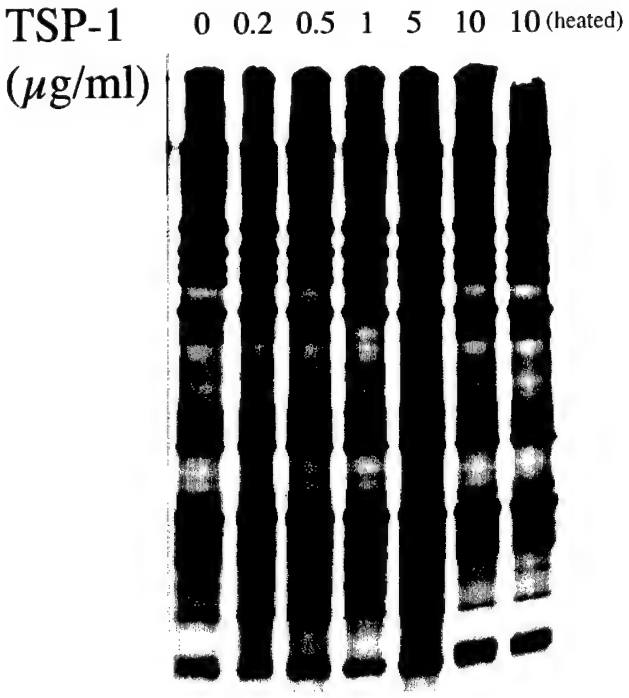
Figure 2. Adhesion of endothelial cells on an $\alpha 3\beta 1$ integrin-binding peptide from TSP1. Panel A: TSP1 peptide 678 (FQGVLQNVRFVF) or analogs of this peptide with the indicated Ala substitutions (*) were absorbed on bacteriological polystyrene substrates at 10 μM in PBS. Direct adhesion of BAE cells to the absorbed peptides or uncoated substrate (control) are presented as mean $\pm$ SD, n = 3. Panel B: Loss of cell-cell contact stimulates endothelial cell spreading on TSP1. Two flasks of BAE cells were grown to confluence. One flask was harvested and replated in fresh medium at 25% confluency. Fresh medium was added at the same time to the second flask. After 16 h, cells from both flasks were dissociated using EDTA, and adhesion was measured on substrates coated with 40 μg/ml TSP1, 10 μg/ml vitronectin, 20 μg/ml plasma fibronectin, or 5 μg/ml type I collagen. The percent spread cells from confluent (solid bars) or sparse cultures (striped bars) after 60 min. is presented as mean $\pm$ SD, n = 3 for a representative experiment.

Figure 3. Spreading on TSP1 induced by loss of cell-cell contact is inhibited by the $\alpha 3\beta 1$ integrin-binding peptide from TSP1. BAE cells from confluent (a,b) or sparse cultures (c-f) were incubated for 60 min. on substrates coated with 40 μg/ml TSP1 (a, c, e) or 20 μg/ml fibronectin (b, d, f). Inhibition by 30 μM TSP1 peptide 678 is presented in (e-f). Cells were fixed with 1% glutaraldehyde and stained using Diff-quik. Bar in panel a = 25 μm.

Figure 4.. Loss of cell-cell contact induces endothelial cell spreading on TSp1 peptide 678. Panel A: Adhesion of sparse or confluent BAE cells to substrates coated with 40 μg/ml TSP1 (solid bars) or 10 μM TSP1 peptide 678 (striped bars) was determined as in Fig. 1B. Spreading was determined microscopically for cells with no additions, in the presence of 10 μM peptide 678, or in the presence of 30 μM of the control peptide 690. Results are presented as mean ± SD, n = 3. Panel B: HDME cells harvested from confluent or sparse cultures as in Fig. 1 were plated on substrates coated with TSP1 (solid bars), peptide 678 (striped bars), or type I collagen (open bars). The percent spread cells was determined at 60 min.

Figure 5.. Both $\alpha 3\beta 1$ and $\alpha v\beta 3$ integrins mediate spreading of endothelial cells on thrombospondin-1. Panel A: BAE cell adhesion to TSP1 (solid bars), vitronectin (striped bars), or plasma fibronectin (open bars) was measured in the presence of 30 μM TSP1 peptide 678, 1 μM of the $\alpha v\beta 3$ integrin antagonist SB223245, 300 μM of the integrin antagonist peptide GRGDSP, or the indicated combinations. Results are expressed as percent of the response for untreated cells, mean ± S.D., n = 3. Panel B: HUVEC spreading on substrates coated with TSP1 (solid bars) or vitronectin (striped bars) was determined in the presence of 20 μM peptide 678, 1 μM $\alpha IIb\beta 3$ antagonist SB208651, 1 μM $\alpha v\beta 3$ antagonist SB223245, or 20 μM peptide 678 plus 1 μM SB223245. Spreading is presented as a percent of the respective controls without inhibitors (31 cells/mm^2 for TSP1 and 10 cells/mm^2 for vitronectin). Panel C: inhibition of HDME cell spreading on TSP1 (solid bars) or type I collagen (striped bars) was determined in the presence of the indicated function blocking antibodies at 5 μg/ml: anti-CD36 (OKM5), anti-integrin $\beta 1$ (mAb13), anti-integrin $\alpha 3$ (P1B5), and anti-integrin $\alpha 4$ (P4C2).

FIGURE 1



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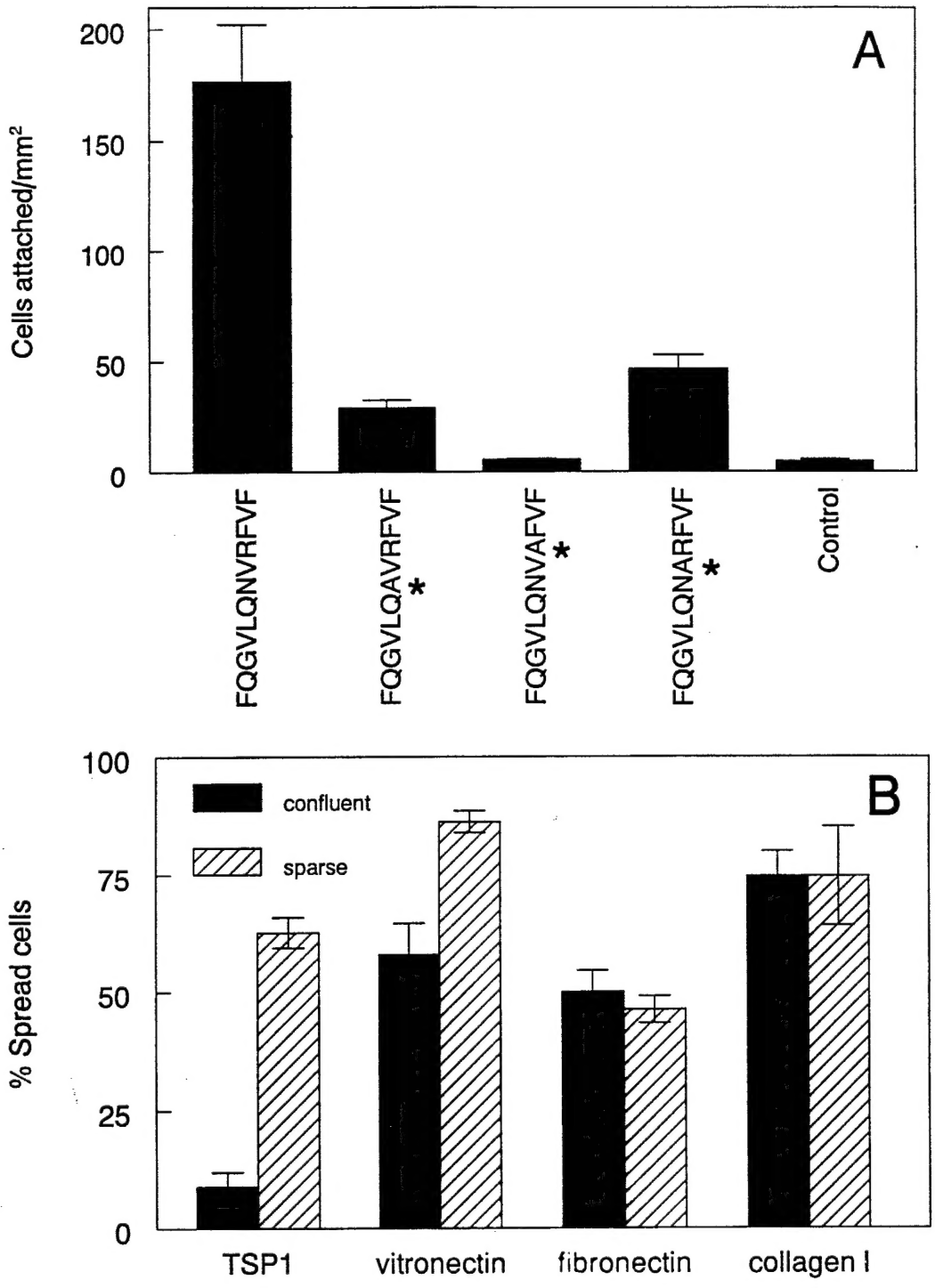
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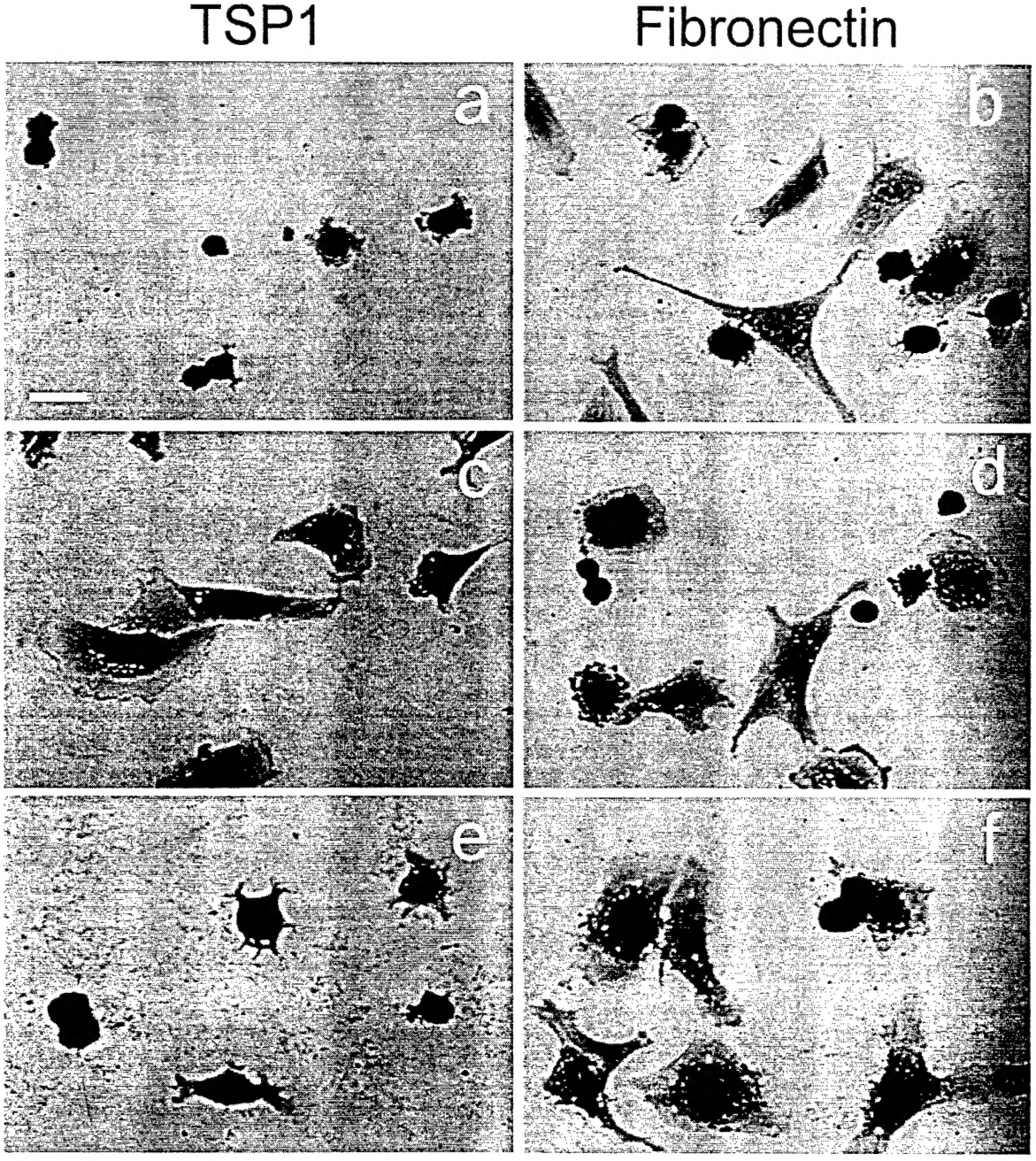
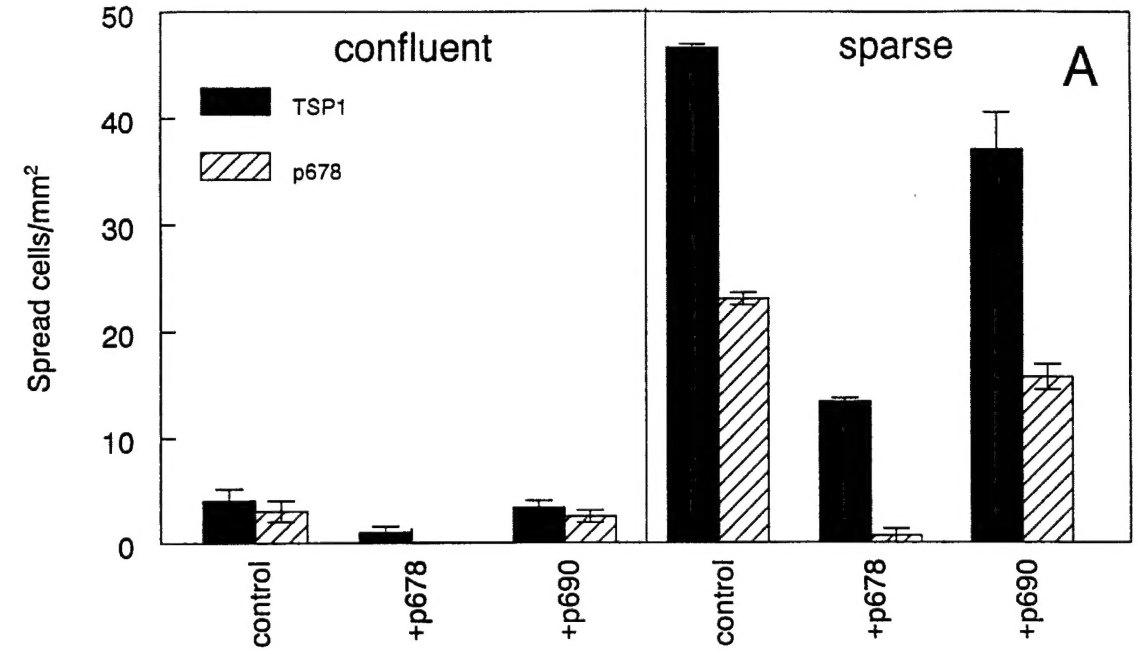


FIGURE 3

FIGURE 4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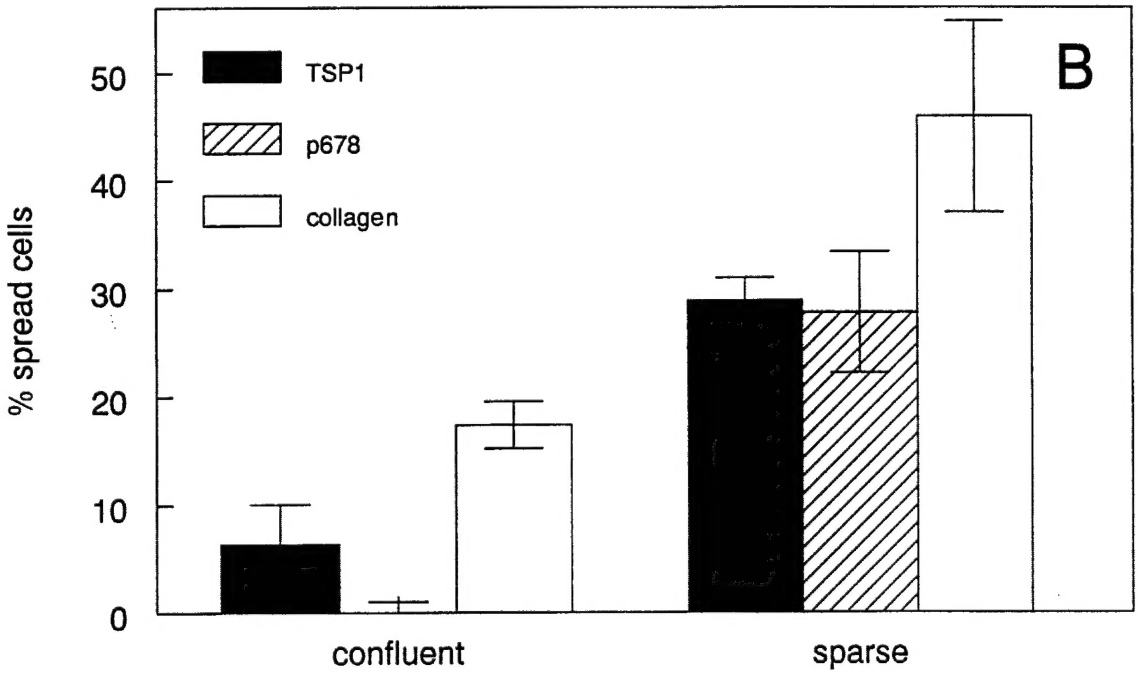
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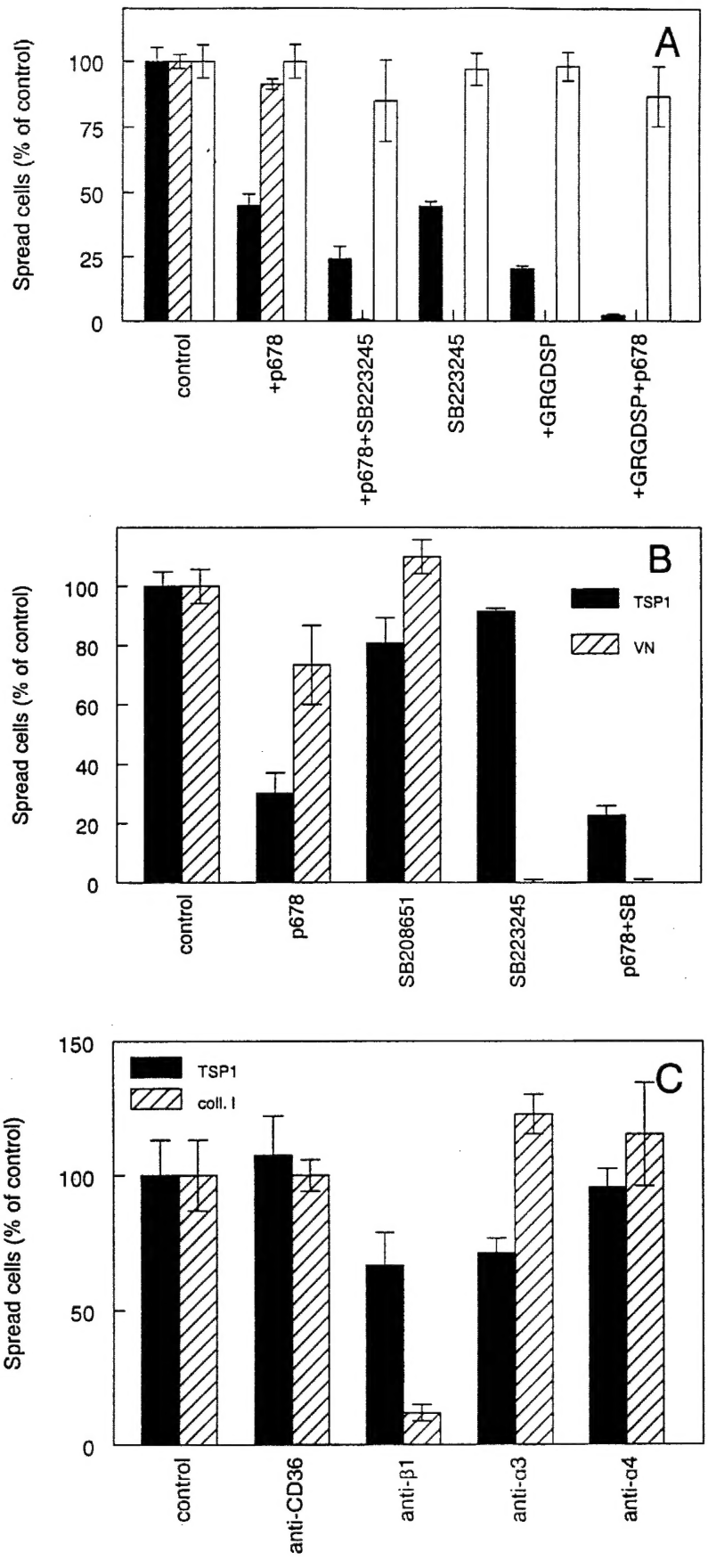
FIGURE 5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50
25
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control
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B
Spread cells (% of control)
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60
40
20
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control
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100
50
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anti-α3
anti-α4